



Supplementary Materials for

An orally active small molecule fusion inhibitor of influenza virus

Maria J.P. van Dongen^{1, 2 *§}, Rameshwar U. Kadam^{3§}, Jarek Juraszek¹, Edward Lawson⁴, Boerries Brandenburg^{1, 5}, Frederike Schmitz¹, Wim B.G. Schepens², Bart Stoops², Harry A. van Diepen¹, Mandy Jongeneelen^{1, 5}, Chan Tang^{1, 5}, Jan Vermond¹, Alida van Eijgen-Obregoso Real¹, Sven Blokland^{1, 5}, Divita Garg⁶, Wenli Wu³, Wouter Goutier¹, Ellen Lanckacker⁷, Jaco M. Klap¹, Daniëlle C. G. Peeters², Jin Wu⁷, Christophe Buyck², Tim H. M. Jonckers⁷, Dirk Roymans⁷, Peter Roevens², Ronald Vogels^{1, 5}, Wouter Koudstaal^{1†}, Robert H. E. Friesen^{1††}, Pierre Raboisson^{7¶}, Dashyant Dhanak^{2, 4#}, Jaap Goudsmit^{1, 8} and Ian A. Wilson^{3, 9 *}

Corresponding to: mvandon@its.jnj.com (M.J.P.v.D.); wilson@scripps.edu (I.A.W.)

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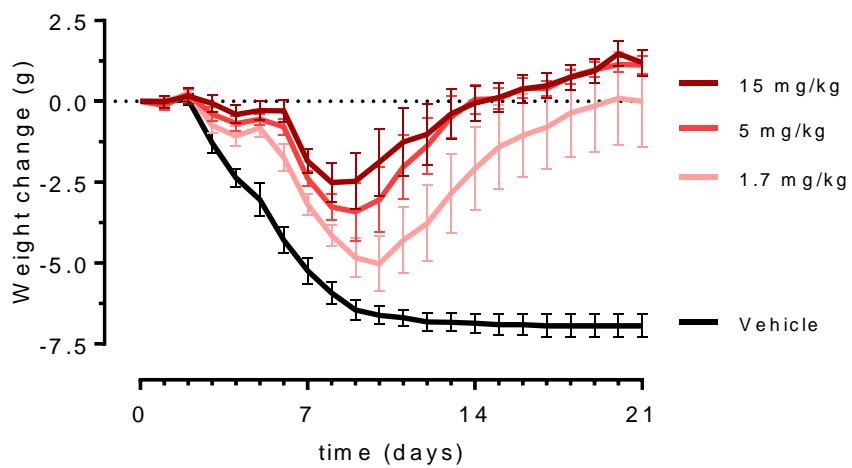
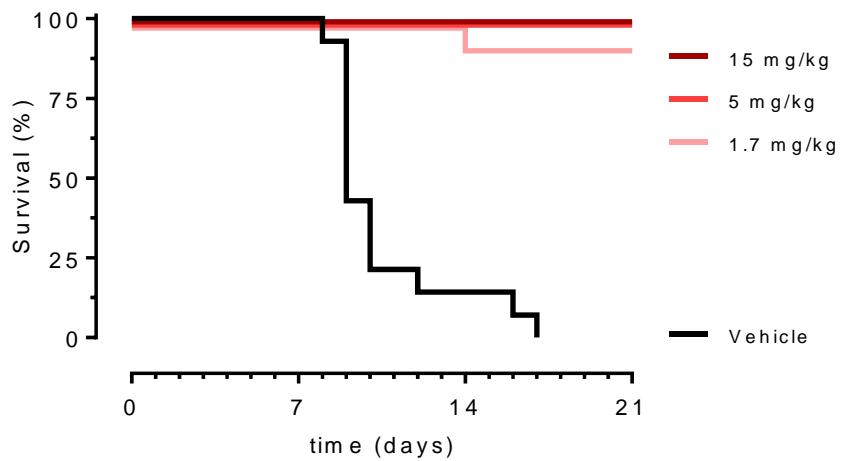


Fig. S1. Survival curves and weight loss of sub-lethal challenged mice. Mice were challenged intranasally (at day 0) with a sublethal dose of the mouse-adapted H1N1 A/PR/8/1934 virus (MLD₉₀) after oral administration of the indicated compounds at day -1 to 5 (twice daily).

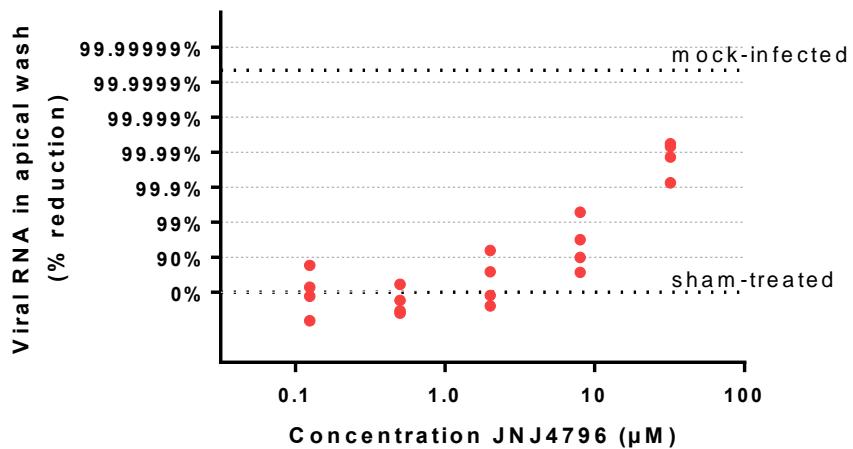


Fig. S2. Reduction of viral titers in human bronchial epithelial cell culture. Cells were infected with H1N1 A/PR/8/1934 virus and concomitantly treated with JNJ4796 at the indicated concentrations. Viral titers 96 hours post-infection were via measurement of viral RNA as determined by qRT-PCR. P-values for the three highest concentration relative to the lower plateau are 0.45, 0.0006 and <0.0001, respectively.

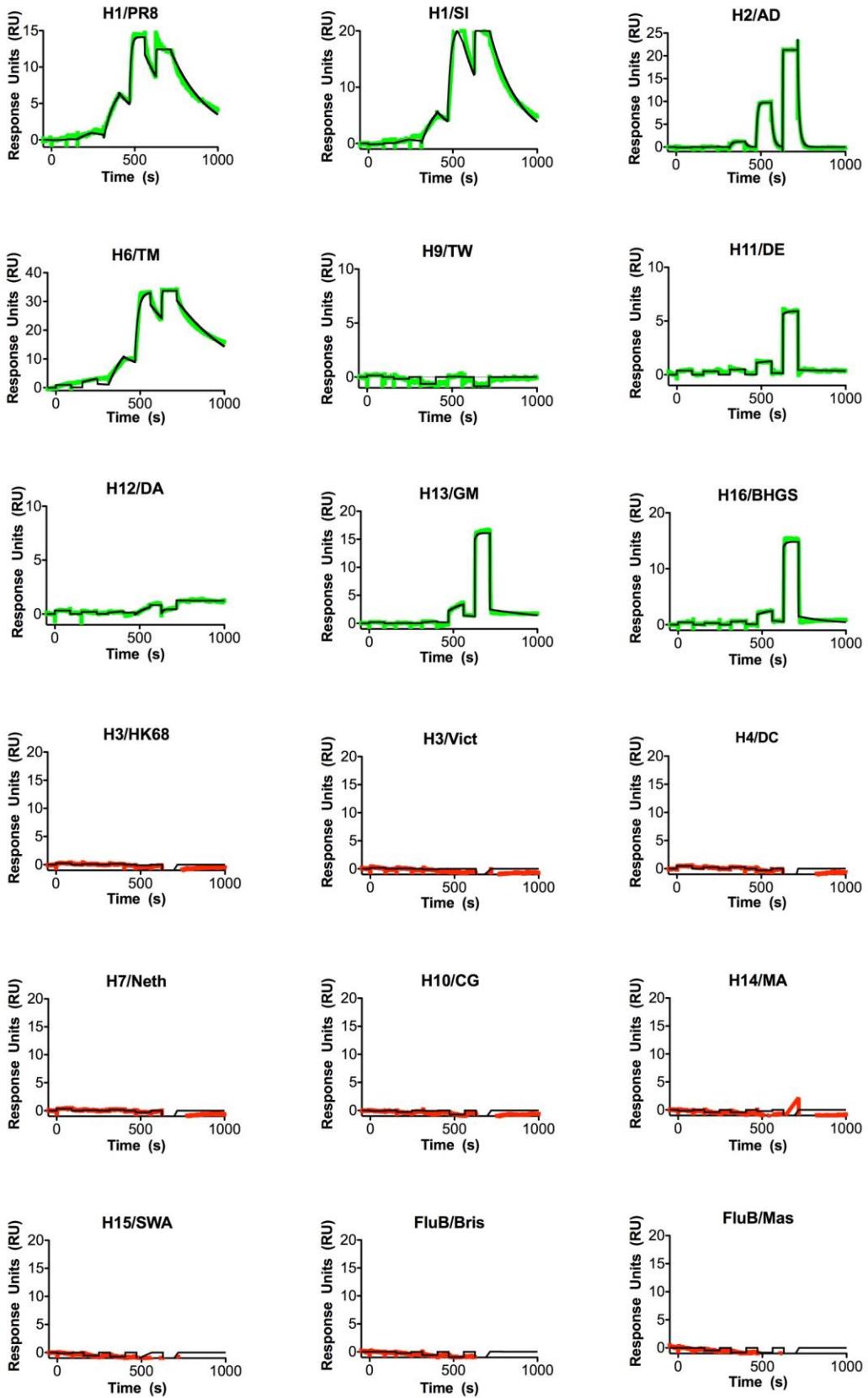


Fig. S3. Surface plasmon resonance (SPR) sensorgrams for HA-JNJ4796 binding. Single-cycle kinetics were run on a Biacore T200 to investigate the binding kinetics of JNJ4796 against influenza A and B HAs. Group-1 HAs: H1/PR8 = A/Puerto Rico/8/1934, H1/SI = A/Solomon Islands/3/2006, H2/AD = A/Adachi/2/1957, H6/TM = A/turkey/Massachusetts/3740/ 1965, H9/TW = A/turkey/Wisconsin /1/1966, H11/DE = A/duck/ England /1/1956, H12/DA = A/duck/Alberta/ 60/1976, H13/GM = A/gull/Maryland/704/1977, H16/BS = A/black-headed gull/Sweden /4/1999. Group-2 HAs: H3/HK68 = A/Hong Kong/1/1968, H3/Vict = A/Victoria/361/2011, H4/DC = A/duck/Czechoslovakia/1956, H7/Neth = A/Netherlands/219/2003, H10/CG = A/chicken/Germany/N/1949, H14/MA = A/mallard/Astrakhan/263/1982, H15/SWA = A/shearwater/W. Australia/2576/79. Influenza B HAs: FluB/Bris = B/Brisbane/33/2008 and FluB/Mas = B/Massachusetts/02/2012. JNJ4796 from 0.1 nM to 1 μ M was passed over the immobilized HAs. Sensorgrams in response units (RU) plotted against time of injection are shown. Curves are the experimental trace obtained from SPR experiments are represented in green (influenza A group-1) and in red [influenza A group-2 and influenza-B (flu-B)]. The overlaid black lines are the best global fits (1:1 Langmuir binding model) to the data used to calculate the association rate constants (ka) and dissociation rate constants (kd). The analysis is summarized in table S5.

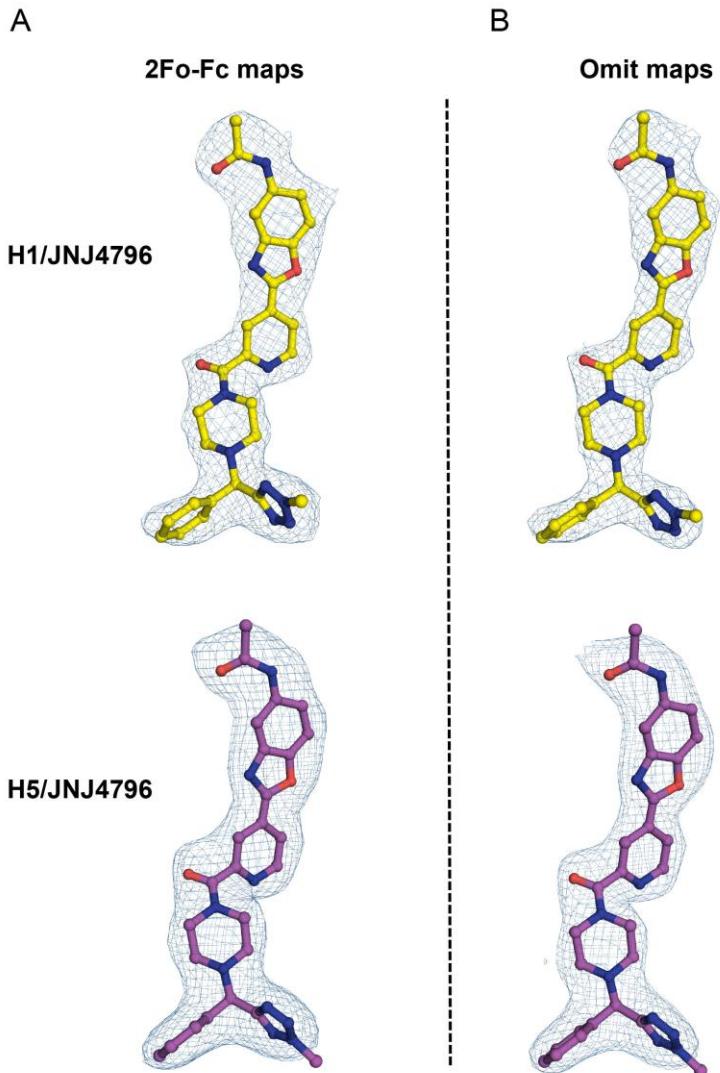


Fig. S4. Electron density maps for JNJ4796 in complex with H1/SI06 and H5/Viet HAs. (A) 2Fo-Fc and (B) simulated annealing (SA) omit maps, contoured at 1σ . For JNJ4796 in complex with H1/SI06, C/O/N are represented in yellow/red/blue sticks, whereas in complex with H5/Viet04, C/O/N are represented in magenta/red/blue sticks, respectively. Electron density maps are represented in a skyblue mesh.

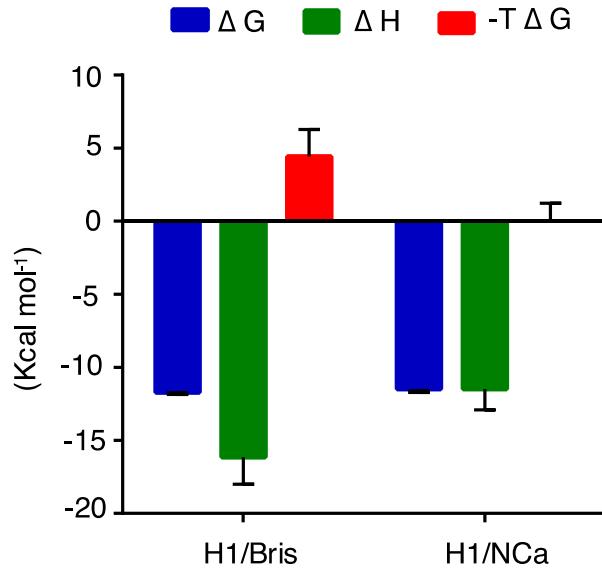


Fig. S5. Thermodynamic profile of JNJ4796 with influenza HA. Binding energy of JNJ4796 (y-axis in kcal per mole) against H1N1 A/Brisbane/59/2007 (H1/Br/07) and A/New Caledonia/20/1999 (H1/NCA) HAs (x-axis). The free energy of binding (ΔG) in blue, enthalpy (ΔH) in green, and entropy ($-T\Delta S$) in red.

Table S1. *In vitro* cytotoxicity data for small molecule compounds

Cytotoxicity EC ₅₀ (μM)	Small molecule compounds			
	JNJ7918	JNJ6715	JNJ8897	JNJ4796
	16.60	>50	>10	>10

Table S2. Selectivity data for JNJ4796. Competition binding utilizing radioactive-labeled reference compound

Assay	% inhibition*	
A1 (h) (antagonist radioligand)	16.8	delta (DOP) (h) (agonist radioligand)
A2A (h) (agonist radioligand)	-	kappa (KOP) (agonist radioligand)
A3 (h) (agonist radioligand)	16.1	mu (MOP) (h) (agonist radioligand)
alpha 1 (non-selective) (antagonist radioligand)	12.3	NOP (ORL1) (h) (agonist radioligand)
alpha 2 (non-selective) (antagonist radioligand)	-	5-HT1A (h) (agonist radioligand)
beta 1 (h) (agonist radioligand)	-	5-HT1B (antagonist radioligand)
AT1 (h) (antagonist radioligand)	-	5-HT2A (h) (antagonist radioligand)
BZD (central) (agonist radioligand)	-	5-HT2B (h) (agonist radioligand)
B2 (h) (agonist radioligand)	-	5-HT3 (h) (antagonist radioligand)
CCK1 (CCKA) (h) (agonist radioligand)	-	5-HT5a (h) (agonist radioligand)
D1 (h) (antagonist radioligand)	-	5-HT6 (h) (agonist radioligand)
D2S (h) (antagonist radioligand)	21.8	5-HT7 (h) (agonist radioligand)
ETA (h) (agonist radioligand)	-	sst (non-selective) (agonist radioligand)
GABA (non-selective) (agonist radioligand)	-	VPAC1 (VIP1) (h) (agonist radioligand)
GAL2 (h) (agonist radioligand)	-	V1a (h) (agonist radioligand)
CXCR2 (IL-8B) (h) (agonist radioligand)	-	Ca2+ channel (L, verapamil site) (phenylalkylamine) (antagonist radioligand)
CCR1 (h) (agonist radioligand)	-	KV channel (antagonist radioligand)
H1 (h) (antagonist radioligand)	-	SKCa channel (antagonist radioligand)
H2 (h) (antagonist radioligand)	-	Na+ channel (site 2) (antagonist radioligand)
MC4 (h) (agonist radioligand)	-	Cl- channel (GABA-gated) (antagonist radioligand)
MT1 (ML1A) (h) (agonist radioligand)	27.5	norepinephrine transporter (h) (antagonist radioligand)
M1 (h) (antagonist radioligand)	-	dopamine transporter (h) (antagonist radioligand)
M2 (h) (antagonist radioligand)	-	5-HT transporter (h) (antagonist radioligand)
M3 (h) (antagonist radioligand)	-	
NK2 (h) (agonist radioligand)	18.4	
NK3 (h) (antagonist radioligand)	10.2	
Y1 (h) (agonist radioligand)	-	
Y2 (h) (agonist radioligand)	-	
NTS1 (NT1) (h) (agonist radioligand)	-	
Assay	% inhibition*	

* Results are expressed as a percent inhibition of control specific binding (mean values; n=2) in the presence of 10 μ M JNJ4796; ‘-‘ indicates an inhibition of less than 10%.

Table S3. Mouse survival at day 21 after challenge with H1N1 A/PR/8/34 virus. Groups receiving SMs p.o. from day -1 to 5 were compared with the p.o. vehicle control group

Viral challenge dose	Treatment	Administration	Route	Survival (%)	P-value (treatment vs control)
25 x MLD50	10 mg/kg JNJ8897	Day -1 to 5	p.o.	25	0.732
25 x MLD50	50 mg/kg JNJ8897	Day -1 to 5	p.o.	40	0.347
25 x MLD50	vehicle for JNJ8897	Day -1 to 5	p.o.	0	-
25 x MLD50	10 mg/kg JNJ4796	Day -1 to 5	p.o.	100	<0.001
25 x MLD50	50 mg/kg JNJ4796	Day -1 to 5	p.o.	100	<0.001
25 x MLD50	vehicle for JNJ4796	Day -1 to 5	p.o.	0	-
MLD90	1.7 mg/kg JNJ4796	Day -1 to 5	p.o.	92.6	<0.001
MLD90	5 mg/kg JNJ4796	Day -1 to 5	p.o.	100	<0.001
MLD90	15 mg/kg JNJ4796	Day -1 to 5	p.o.	100	<0.001
MLD90	vehicle for JNJ4796	Day -1 to 5	p.o.	0	-

Table S4. Weight loss of mice as area under the curve (AUC) reflected as change from baseline after challenge with H1N1 A/PR/8/34 virus.

Viral challenge dose	Treatment	Administration	Route	Mean AUC (g*day)	SD (g*day)
25 x MLD50	10 mg/kg JNJ8897	Day -1 to 5	p.o.	-70.87	36.97
25 x MLD50	50 mg/kg JNJ8897	Day -1 to 5	p.o.	-63.09	31.51
25 x MLD50	vehicle for JNJ8897	Day -1 to 5	p.o.	-92.85	13.32
25 x MLD50	10 mg/kg JNJ4796	Day -1 to 5	p.o.	-47.14	9.68
25 x MLD50	50 mg/kg JNJ4796	Day -1 to 5	p.o.	-22.47	11.92
25 x MLD50	vehicle for JNJ4796	Day -1 to 5	p.o.	-89.71	9.81
MLD90	1.7 mg/kg JNJ4796	Day -1 to 5	p.o.	-38.00	25.53
MLD90	5 mg/kg JNJ4796	Day -1 to 5	p.o.	-13.90	11.97
MLD90	15 mg/kg JNJ4796	Day -1 to 5	p.o.	-7.52	15.85
MLD90	vehicle for JNJ4796	Day -1 to 5	p.o.	-107.36	8.06

Table S5. SPR kinetic data for JNJ4796 binding to influenza A and B HAs.

Influenza A	Lineage	Subtype	Strain	K _D (nM)*
Influenza A	Group 1	H1N1	A/Puerto Rico/8/1934	4.2
		H1N1	A/Solomon Islands/3/2006	12.2
		H2N2	A/Adachi/2/1957	195.1
		H6N2	A/turkey/Massachusetts/3740/1965	5.2
		H9N2	A/turkey/Wisconsin/1/1966	N.B.
		H11N6	A/duck/England/1/1956	17.4
		H12N5	A/duck/Alberta/60/1976	N.B.
		H13N6	A/gull/Maryland/704/1977	20.1
		H16N3	A/black-headed gull/Sweden/4/1999	49.4
Influenza A	Group 2	H3N2	A/Hong Kong/1/1968	N.B.
		H3N2	A/Victoria/361/2011	N.B.
		H4N6	A/duck/Czechoslovakia/1956	N.B.
		H7N7	A/Netherlands/219/2003	N.B.
		H10N7	A/chicken/Germany/N/1949	N.B.
		H14N5	A/mallard/Astrakhan/263/1982	N.B.
		H15N9	A/shearwater/W. Australia/2576/79	N.B.
Influenza B	Victoria	-	B/Brisbane/33/2008	N.B.
	Yamagata	-	B/Massachusetts/02/2012	N.B.

* Values from single experiments; N.B., no binding

Table S6. Data collection and refinement statistics for JNJ4796 complexes with H1 and H5 HAs

	JNJ4796-H1/SI06	JNJ4796-H5/Viet
Data collection		
Beamline	SSRL 12-2	SSRL 12-2
Wavelength (Å)	0.97946	0.97946
Space Group	P4 ₁ 32	H32
Unit cell (Å, °)	a=b=c=161.4	a=b=102.1, c=454.0
Resolution range (Å) ^a	50-2.72 (2.77-2.72)	50-2.32 (2.36-2.32)
Observations	246,263	396,124
Unique reflections	19,893 (967)	40,297 (1773)
Completeness (%)	99.6 (100)	99.2 (89.5)
I/σ(I)	22.4 (2.1)	26.1 (1.1)
R _{sym} ^b	0.10 (0.86)	0.08 (0.77)
R _{pim} ^c	0.03 (0.24)	0.02 (0.28)
CC _{1/2} ^d	0.97 (0.84)	0.97 (0.88)
Redundancy	12.4 (13.0)	9.8 (6.1)
Refinement		
Resolution (Å)	44.78-2.72	40.40-2.32
No. reflections ^e	19,834 (996)	39,877 (2036)
R _{cryst} ^f /R _{free} ^g	0.23/0.25	0.20/0.24
Protein atoms	3905	4009
Ligand/Glycan atoms	40/212	40/84
Waters	52	100
Wilson B (Å ²)	59	46
Average B value (Å ²)		
Proteins	72	71
Ligand	52	63
Water	54	63
RMSD from ideal geometry		
Bond length (Å)	0.004	0.008
Bond angle (°)	0.6	0.9
Ramachandran Statistics (%) ^h		
Favored	96.9	96.4
Outliers	0	0
PDB codes	6CF7	6CFG

^aParentheses refer to outer shell statistics.

^b $R_{\text{sym}} = \sum_{hkl} \sum_i |I_{hkl,i} - \langle I_{hkl} \rangle| / \sum_{hkl} \sum_i I_{hkl,i}$, where $I_{hkl,i}$ is the scaled intensity of the i^{th} measurement of reflection h, k, l , and $\langle I_{hkl} \rangle$ is the average intensity for that reflection.

^c $R_{\text{pim}} = \sum_{hkl} (1/(n-1))^{1/2} \sum_i |I_{hkl,i} - \langle I_{hkl} \rangle| / \sum_{hkl} \sum_i I_{hkl,i}$, where n is the redundancy.

^d $CC_{1/2}$ = Pearson Correlation Coefficient between two random half datasets.

^eValue in the parentheses refers to number of reflections in R_{free} test set.

^f $R_{\text{cryst}} = \sum_{hkl} |F_o - F_c| / \sum_{hkl} |F_o| \times 100$, where F_o and F_c are the observed and calculated structures factors.

^g R_{free} was calculated as for R_{cryst} , but on a test set of 5% of the data excluded from refinement.

^hCalculated using MolProbity.